selected from the group consisting of: PGU1, FLO11, TOT10/YEL033W, SRD1, TOT12/YKR105C, TOT13/YOR225W, FLO5, DDR48, TOT11/YLR042C, TOT7/YER158C, TOT8/YIL117C, TOT20/YHL049C, TOT15/YLR434C, TOT14/YBR113W, TOT9/YIR013C, PHO84, KTR2, and SJH1;

b) contacting said host cell with an agent to be tested; and

c) comparing the expression of said gene in the presence of the agent with the expression of said gene in the absence of said agent, wherein a difference in the expression of said gene in the presence of the agent as compared with in the absence of the agent indicates that the agent modulates expression of said gene which is modulated by the filamentation MAPK pathway in a fungus.

Amendments to the claims are indicated in the attached "Marked Up Version of Amendments" (pages i-ii).

REMARKS

Claim Amendments

Claims 9, 15 and 19 have been amended to more clearly define the invention. Specifically, Claims 9, 15 and 19 have been amended to recite a host cell capable of expressing a gene when transformed with an expression vector. Claim 19 has also been amended to recite a host cell expressing a gene identified in the Specification. Support for the amendments can be found in the Specification, for example on page 6, lines 3-5, page 9, lines 3-12 and Figure 2.

No new matter has been added by these amendments.

Rejection of Claims 9, 11 and 15 under 35 U.S.C. § 112, First paragraph

Claims 9, 11 and 15 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The Examiner states on page 3 of the Office Action that the application discloses methods which are dependent upon expression of genes encoding TOT10/YEL033W or PGU1

and that neither of these gene sequences is disclosed in the specification. The Examiner also states that these yeast strains from which PGU1 and TOT10/YEL033W gene sequences were obtained are not clearly described in the specification, and that, because it is apparent that this biological material is essential for practicing the claimed invention, it must be obtainable by a reproducible method set forth in the specification or otherwise be known and readily available to the public, as detailed in 37 C.F.R. §§ 1.801-1.809. The Examiner further states that it is unclear whether this biological material is known and readily available to the public, or that the written instructions are sufficient to reproducibly construct this biological material from starting materials known and readily available to the public. The Examiner concludes that if this biological material is not obtainable or available, the requirements of 35 U.S.C. § 112 may be satisfied by a deposit of the biological material.

Respectfully, Applicant does not agree. The DNA sequence for all genes disclosed in the specification were readily identifiable by the skilled artisan from yeast genome databases such as http://genome-www.stanford.edu/Saccharomyces/ by searching using the gene names disclosed in the subject application. For example, Applicant has attached the results of a database search for YEL033W and PGU1 (Exhibit A). Applicant affirms that this information was available from http://genome-www.stanford.edu/Saccharomyces/ at the time of filing.

Applicant also directs the Examiner to Blanco *et al.* (*FEMS Microbiol. Lett 164*:249-255, (1998)) a copy of which is attached (Exhibit B), which teaches the sequence of PGU1 and that PGU1 is detected in all yeast strains regardless of their phenotype (page 249, Abstract). Furthermore, Blanco *et al.* teach that polygalacturonases have been reported in plants, filamentous fungi, yeasts and bacteria (page 249).

Thus, the genes used in Applicant's invention were commonly known and available to the public at the time of filing. Therefore, the Specification in combination with the knowledge of the skilled artisan enabled one of skill in the art to make and/or use the invention at the time of filing as required under 35 U.S.C. 112, first paragraph. Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claim 19 Under 35 U.S.C. § 112, First Paragraph

Claim 19 is rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Examiner states on pages 4 and 5 that Claim 19 is drawn to a method of identifying an agent which modulates expression of a gene which is modulated by the MAPK pathway and that the phrase "a gene which is modulated by the MAPK pathway" is not clearly defined in the specification. The Examiner concludes that given the large genus of different MAPK pathways from different organisms encompassed by Claim 19, the even larger number of genes which can be interpreted as "modulated" by those MAPK pathways, and the lack of descriptions of the genes or response elements involved in any MAPK pathway from any source other than for the Kss1/Tec1-responsive pathways in yeast, one of ordinary skill in the art would not be able to envision a representative number of different embodiments of the claims genus based upon the descriptions in the specification.

Respectfully, Applicant disagrees. However, solely to advance prosecution, Claim 19 has been amended to recite transforming a suitable host cell capable of expressing a gene when transformed with an expression vector comprising a nucleic acid molecule encoding a gene selected from the group consisting of: PGU1, FLO11, TOT10/YEL033W, SRD1, TOT12/YKR105C, TOT13/YOR225W, FLO5, DDR48, TOT11/YLR042C, TOT7/YER158C, TOT8/YIL117C, TOT20/YHL049C, TOT15/YLR434C, TOT14/YBR113W, TOT9/YIR013C, PHO84, KTR2, and SJH1. Applicant believes the amendment to Claim 19 obviates the Examiner's rejection. Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 9, 11, 15 and 19 Under 35 U.S.C. § 112, Second Paragraph

Claims 9, 11, 15 and 19 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

The Examiner states on page 6 that Claims 9, 15 and 19 are vague and indefinite and that the metes and bounds of the term "suitable host cell" are unclear. The Examiner also states that

this term is inherently indefinite and that it would be remedial to amend the claim language to clearly indicate what constitutes a "suitable host cell."

Applicant has amended Claims 9, 15 and 19 to indicate that a suitable host cell is capable of expressing a gene when transformed with a nucleic acid molecule encoding a gene.

The Examiner states that Claim 19 is vague and indefinite in that the metes and bounds of the phrase "a gene which is modulated by the MAPK pathway" are unclear.

Claim 19, as amended, recites the "filamentation" MAPK pathway and also recites specific genes which have been identified by Applicant as modulated by the filamentation MAPK pathway. Applicant believes Claim 19, as amended, even more particularly points out and distinctly claims the subject matter which Applicant regards as the invention. Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claim 19 Under 35 U.S.C. §102(b)

Claim 19 is rejected under 35 U.S.C. §102(b) as being anticipated by Madhani, *et al.* (Science 2/28/97, Vol. 275, pages 1314-1317; see entire document). The Examiner states that Madhani *et al.* describe the construction of reporter genes under the control of promoter/regulatory sequences which depend specifically on the MAPK signaling components of yeast that promote filamentous, an invasive growth. The Examiner concludes that it was demonstrated that for yeast cells comprising the FRE(Tec1):: lacZ reporter gene, expression of the reporter increased significantly upon expression of proteins STE11-4 or the TEC1 from high-copy plasmids introduced into cells (e.g., Figure 3).

Claim 19 has been amended herein to recite transforming a suitable host cell capable of expressing a gene when transformed with an expression vector comprising a nucleic acid molecule encoding a gene selected from the group consisting of: PGU1, FLO11, TOT10/YEL033W, SRD1, TOT12/YKR105C, TOT13/YOR225W, FLO5, DDR48, TOT11/YLR042C, TOT7/YER158C, TOT8/YIL117C, TOT20/YHL049C, TOT15/YLR434C, TOT14/YBR113W, TOT9/YIR013C, PHO84, KTR2, and SJH1. Madhani *et al.* do not teach or suggest any of these particular genes or their modulation by a filamentation MAPK pathway in a fungus. Madhani *et al.* do not teach or suggest every aspect of Applicant's invention. Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned at (781) 861-6240.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Pamela A. Torpey (45,736) for

Lisa M. Treannie
Registration No. 41,368
Telephone (781) 861-6240
Facsimile (781) 861-9540

Lexington, Massachusetts 02421-4799
Dated: September 27, 2001

MARKED UP VERSION OF AMENDMENTS

Claim Amendments Under 37 C.F.R. § 1.121(c)(1)(ii)

9. (Twice Amended) A method of identifying an agent which inhibits the filamentation MAPK pathway in a fungus, comprising the steps of:

a) transforming a suitable host cell capable of expressing a gene when transformed with an expression vector comprising a nucleic acid molecule encoding TOT10/YEL033W under conditions suitable for expression of TOT10/YEL033W;

b) contacting said host cell with an agent to be tested; and

c) comparing the expression of TOT10/YEL033W in the presence of the agent with the expression of TOT10/YEL033W in the absence of said agent, wherein if the expression of TOT10/YEL033W is lower in the presence of the agent than in the absence of the agent, then the agent is an inhibitor of the filamentation MAPK pathway in a fungus.

15. (Twice Amended) A method of identifying an agent which modulates PGUI gene expression, comprising the steps of:

a) transforming a suitable host cell capable of expressing a gene when transformed with an expression vector comprising a nucleic acid molecule encoding PGUI under conditions suitable for expression of PGUI;

b) contacting said host cell with an agent to be tested; and

c) comparing the expression of PGUI in the presence of the agent with the expression of PGUI in the absence of said agent, wherein a difference in the expression of PGUI in the presence of the agent as compared with in the absence of the agent indicates that the agent modulates PGUI expression.

19. (Amended) A method of identifying an agent which modulates expression of a gene which is modulated by [the] a filamentation MAPK pathway in a fungus, comprising the steps of:

a) transforming a suitable host cell capable of expressing a gene when transformed with an expression vector comprising a nucleic acid molecule encoding a gene selected from the group consisting of: PGU1, FLO11, TOT10/YEL033W, SRD1,

TOT12/YKR105C, TOT13/YOR225W, FLO5, DDR48, TOT11/YLR042C, TOT7/YER158C, TOT8/YIL117C, TOT20/YHL049C, TOT15/YLR434C, TOT14/YBR113W, TOT9/YIR013C, PHO84, KTR2, and SJH1 [which is modulated by the filamentation MAPK pathway under conditions suitable for expression of said gene];

b) contacting said host cell with an agent to be tested; and

c) comparing the expression of said gene in the presence of the agent with the expression of said gene in the absence of said agent, wherein a difference in the expression of said gene in the presence of the agent as compared with in the absence of the agent indicates that the agent modulates expression of a gene which is modulated by the filamentation MAPK pathway in a fungus.

EXHIBIT
tabbies
A

YEL033W

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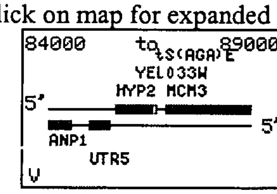
YEL033W BASIC INFORMATION

Systematic Name	YEL033W
Feature Type	ORF
GO Molecular Function	• molecular_function unknown
GO Biological Process	• biological_process unknown
Description	Hypothetical ORF
Phenotype	• Systematic deletion: viable ***More Phenotype Details for YEL033W***
Position	ChrV: coordinates 86179 to 86598 Old format Sequence details
External Links	MIPS | YPD | SwissProt | Entrez Protein | Entrez Neighbors | PIR-DE | PIR-JP | PIR-US | Entrez RefSeq
Primary SGDID	S0000759

YEL033W RESOURCES

Click on map for expanded view



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ADDITIONAL INFORMATION for *YEL033W*

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PGU1/YJR153W

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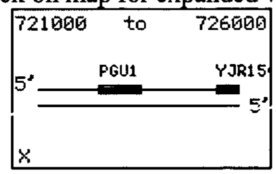
PGU1 BASIC INFORMATION

Standard Name	*PGU1*
Alias	*PGL1, PSM1*
Systematic Name	YJR153W
Feature Type	ORF
GO Annotations	***PGU1 GO evidence and references***
Molecular Function	• polygalacturonase
Description	Endo-polygalacturonase
Gene Product	endo-polygalacturonase
Phenotype	• Old format: Null mutant is viable; exhibits clear halo around colonies on polygalacturonate medium • Systematic deletion: viable
	More Phenotype Details for PGU1
Position	ChrX: coordinates 722508 to 723593 Old format Sequence details
External Links	MIPS | YPD | Entrez Protein | Entrez Neighbors | PIR-DE | PIR-JP | PIR-US | Entrez RefSeq | SwissProt | NiceZyme | Kyoto
Primary SGDID	S0003914

PGU1 RESOURCES

Click on map for expanded view



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ADDITIONAL INFORMATION for *PGU1*

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FEMS Microbiology Letters 164 (1998) 249–255

FEMS MICROBIOLOGY LETTERS

Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b

Pilar Blanco, C. Sieiro, N.M. Reboredo, T.G. Villa *

Department of Microbiology, Faculty of Pharmacy, University of Santiago, Santiago de Compostela, 15706, Spain

Received 17 April 1998; revised 9 May 1998; accepted 14 May 1998

Abstract

A structural polygalacturonase-encoding gene (*PGU1*) from *Saccharomyces cerevisiae* IM1-8b was cloned and sequenced. The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18 and two potential glycosylation points in residues 318 and 330. The putative active site is a conserved histidine in position 222. This polygalacturonase showed 54% homology with the fungal ones and only 24% homology with their plant and bacterial counterparts. The gene is present in a single gene copy per haploid genome and it is detected in all strains, regardless of their phenotype. The expression of *PGU1* gene in several strains of *S. cerevisiae* revealed that the polygalacturonase activity depended on the plasmid used and also on the genetic background of each strain but in all cases the enzymatic activity increased.

Keywords: *Saccharomyces cerevisiae*; Polygalacturonase; Polygalacturonase-encoding gene; Protein sequence; Over-expression

1. Introduction

Polygalacturonases (PGs) are pectolytic enzymes which degrade pectic substances by splitting 1,4-α-glycosidic bonds between two galacturonic acid residues [1]. They are classified by activity as endo-PG and exo-PG; the former hydrolyzes polygalacturonic acid in a random fashion, thus releasing oligosaccharidic chains of variable length, whereas the latter catalyzes the release of single galacturonic acid residues starting from the non-reducing end. The production and characterization of PGs has been reported in plants, filamentous fungi, yeasts and bacteria.

These enzymes are involved as virulence determinants in some bacterial plant diseases, especially by soft-rot *Erwinia* species and genes encoding PGs have been isolated and characterized from several strains of *Erwinia carotovora* and *Erwinia chrysanthemi* [2]. A role of PGs and other cell-wall-degrading enzymes has also been invoked for phytopathogenic filamentous fungi. In the case of *Aspergillus niger*, because of its relevance as a producer of extracellular enzymes for the food industry, its molecular biology has been more developed than in other filamentous fungi [3,4].

Polygalacturonase production in yeasts is not so

* Corresponding author. Tel.: +34 (81) 592490; Fax: +34 (81) 592490; E-mail: mpvilla@usc.es

0378-1097/98/$19.00
PII: S0378-1097(98)00220-1

well known and documented as in bacteria and fungi. The presence of this kind of enzymes has been reported in some yeast species including *Saccharomyces fragilis* [5], *Kluyveromyces marxianus* [6], and *Cryptococcus albidus* [7]. Although *Saccharomyces cerevisiae* is an important yeast in the food industry, PG production has only recently been described and characterized in a few strains [8,9].

Until now, the reports on yeast pectinases have focused mainly on the biochemical characterization of the enzymes, but nothing has been published about their genetic determination, except the report by Blanco et al. [10] who described several genes involved in PG production and the presence of several PGs in *S. cerevisiae*. Their work also reported the presence of an open reading frame in the chromosome X of *S. cerevisiae* exhibiting high homology with PG genes of *A. niger*. In the present work we report on the cloning and molecular characterization of an endo-polygalacturonase-encoding gene from *S. cerevisiae* IM1-8b, as well as the over-expression of this gene in several *S. cerevisiae* strains.

2. Materials and methods

2.1. Strains, culture media and plasmids

Escherichia coli DH5α (F^-, *end A1, hsd R17* (r_K^-, m_K^-), *sup E44, thi-1,* λ^-, *rec A1, gyr A96,* Δ *lac U169*) (∅ 80 *lac*ZΔM15) was used as host for cloning. This strain was grown in LB medium [11], supplemented with ampicillin (100 μg ml^{-1}) for plasmid selection. Relevant characteristics and sources of *S. cerevisiae* strains and plasmids employed in the present study are listed in Table 1. YEPD and minimal medium (SD) supplemented with the required amino acids in each case [12] were used for growing yeasts strains. PG production was detected on plates with polygalacturonic acid (PGA) as previously reported by Blanco et al. [8].

2.2. Enzymatic assays

Crude enzyme sample preparation was performed as described elsewhere [8]. PG activity was measured by evaluating the reducing power according to the method of Somogyi [13] as modified by Nelson [14].

2.3. PCR amplification

Polymerase chain reaction (PCR) was carried out to amplify the coding region of the *PGU1* gene using genomic DNA (10–50 ng) from *S. cerevisiae* as the template. Two primers were designed consisting of a *Bam*HI restriction site linked to sequences flanking the ORF YJR153w from *S. cerevisiae* Genome Database: PG-1 (5′-CGCGGATCCATGATTTCTGCT AATTCATTACTTATTT-3′) and PG-1r (5′-CGCG-GATCCTTAACAGCTTGCACCAGATCCAG-3′). These oligonucleotides were previously phosphorylated with T4 Polynucleotide Kinase (Promega). Amplification was performed employing a thermal cycler Gene Cycler® (Bio-Rad) and using a mix of Taq DNA Polymerase (Promega) and *Pfu* Polymerase (Stratagene). Amplification conditions were: (a) 94°C for 2 min, (b) 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, (c) 72°C for 5 min. Amplified products were purified after agarose gel electrophoresis using the Prep-A-Gene® DNA Purification System (Bio-Rad).

2.4. DNA manipulation and molecular cloning

Standard methods for plasmid DNA isolation, restriction and ligation reactions were used [11]. Alternatively plasmid DNA was purified from recombinant clones employing the Wizard® Plus Midipreps-DNA Purification System (Promega). Genomic DNA from *S. cerevisiae* was isolated using the method described by Struhl et al. [15]. Endonucleases and T4 DNA ligase were purchased from Promega. *E. coli* and *S. cerevisiae* transformations were carried out according to the $CaCl_2$ method [11] and the LiAc procedure [16], respectively. The PCR product (*PGU1*) from strain IM1-8b was cloned into the *Eco*RV site of pBluescript SK+ generating pBSK-PGU1 plasmid which was amplified and used as a source for sequencing. The *PGU1* gene from *S. cerevisiae* IM1-8b was cloned into the yeast expression vectors pBEJ16 and pYES2 (Table 1). pBEJ-PGU1 recombinant plasmid was created by cloning the 1.1 kb *Bam*H1 fragment from pBSK-PGU1, containing the *PGU1* gene, into the pBEJ16 previously linearized with *Bgl*II. pYES-PGU1 construct was obtained by ligating the *Xba*I-*Hin*dIII insert recovered from pBSK-PGU1 into the pYES2 vector cleaved with the same enzymes.

2.5. DNA sequencing and sequence analysis

The pBSK-PGU1 was used as a template for sequencing *PGU1* gene. The DNA sequence was determined by the dideoxy chain termination method of Sanger et al. [17], using [^{35}S]dATP (Amersham) and the USB Sequenase^{IM} (Version 2.0) Kit following the manufacturer's instructions. Analysis of DNA sequences was performed using the GCG package (Wisconsin University Biotechnology Center). Search for homologies was carried out using the BLAST [18] or FASTA [19] programmes. The protein sequence prediction and multiple sequence alignments were obtained using Swiss-Prot databases.

2.6. Southern blot analysis

Total genomic DNA digested with *Hin*dIII and separated by electrophoresis (5 μg per lane) was denatured and then transferred to positively charged nylon membranes (Boehringer Mannheim) employing the Pharmacia 2016 Vacugene Vacuum Blotting System. Probes were labelled with digoxygenin using the DIG High DNA Labeling and Detection Kit II (Boehringer Mannheim). Hybridizations were carried out under standard high stringency conditions. The detection of sequences was performed as recommended by the manufacturer.

3. Results and discussion

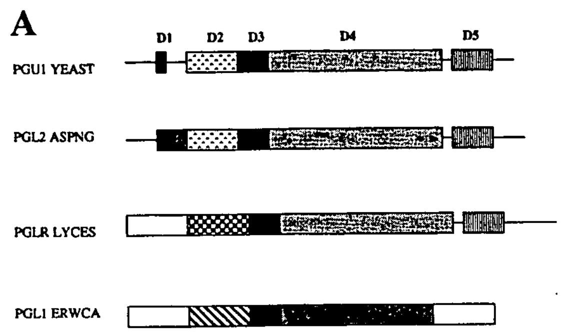
3.1. Cloning and sequencing of a polygalacturonase gene from S. cerevisiae

The biochemical and genetic characterization of PGs produced by *S. cerevisiae* strains 1389 and IM1-8b have been previously described [8–10]. We have reported the existence of different genes involved in PG production in the above strains, although no molecular characterization of the genes was presented. According to the data from the *S. cerevisiae* Genome Database there is an ORF (YJR153w) located on chromosome X that shows high homology with a polygalacturonase-encoding gene from *A. niger* [3]. In order to investigate if that ORF codes for a PG in *S. cerevisiae*, the genomic DNA of the three polygalacturonase positives (PG$^+$) strains (IM1-8b, 1389, and C6) and one polygalacturonase negative (PG$^-$) strain (MI-2B) was used as template for PCR amplification of such gene. The results demonstrated that a single band of ca. 1 kb was amplified from all strains used, re-

Table 1
Strains of *Saccharomyces cerevisiae* and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
S. cerevisiae		
1389	*Wild-type diploid PG*$^+$	CECT
IM1-8b	*MAT*α *leu2-3 leu2-112 his4 PG*$^+$	A. Jimenez[a]
MI-2B	*MAT*α *ura3-52 trp1 PG*$^+$	T. Benítez[b]
C6	*MAT*α *trp1 PG*$^+$	This laboratory
AH22	*MAT*α *leu2-3,112 his4-519 CAN1 PG*$^+$	T. Benítez[b]
STX347-1D	*MAT*α *his2 ura3 gal1 PG*$^+$	YGSC
1389-8b	*MAT*α *ade2 ura3 his7 PG*$^+$	This laboratory
Plasmids		
pBluescript SK+	*Ap*^{R} *β-GAL*	Stratagene
pYES2	*Ap*^{R} *URA3 GALp*	Invitrogen Corporation
pBEJ16	*Ap*^{R} *LEU2 PGKp PGKt*	[21]
PBSK-PGU1	*Ap*^{R} *β-GAL PGU1*	This study
PYES-PGU1	*Ap*^{R} *URA3 GALp PGU1*	This study
PBEJ-PGU1	*Ap*^{R} *LEU2 PGKp PGU1*	This study

CECT, Colección Española de Cultivos Tipo; YGSC, Yeast Genetic Stock Center, Berkeley, CA, USA.
[a]Centro de Biología Molecular, E-28049, Madrid; Spain.
[b]Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Spain.
PG$^+$ and PG$^-$ indicate the ability to hydrolyze or not polygalacturonic acid on plate, respectively.



B

DOMAIN 3

```
PGLR LYCES  169   GGGGTING---NGQVWWPSSCKINK     190
PGU1 YEAST  101     SGHTIDG---QGAKWWDGLGDSG     120
PGL2 ASPNG  103     SGHLINC---DGARWWDGKGTS      121
PGL1 ERWCA  148   QGGVKLQD----KKVSWWDLAADAKV    169
      Consensus     gg   dg     g  wW
```

DOMAIN 4

```
PGLR LYCES  235   SNLMINASA---------------------------KSPNTDGVHVSNT   256
PGL1 ERWCA  214   WKTTIKTPS---------------------------TARNTDGIDPMSS   235
PGL2 ASPNG  162   TDVTINNADGDTQGG-----------------------HNTDAFDVGNS   187
PGU1 YEAST  161   SDITIDIRDGDSAGG-----------------------HNTDGFDVGSS   186
      Consensus       i  p                            s NTDgid    s

PGLR LYCES  257   QYIQISDTIIGTGDDCISIVSG-----SQNVQATNITCGPGHGISIGSLG   301
PGL1 ERWCA  236   KNITIAHSNISTGDDNVAIKAYKGRSETRNISILHNEFGTGHGMSIGSET   285
PGL2 ASPNG  188   VGVNIIKPWVHNQDDCLAVNSG------ENIWFTGGTCIGGHGLSIGSVG   231
PGU1 YEAST  187   SNVLIQGCTVYNQDDCIAVNSG------STIKFMNNYCYNGHGISVGSVG   230
      Consensus     v i    i tgDDc ai  g        ni       cg GHGiSiGSlg

PGLR LYCES  302   SGNSEAYVSNVTVNEAKIIGAENGVRIKTWQGGS--GQASNIKF   343
PGL1 ERWCA  286   -MGVYNVTVDDLIMTGTT----NGLRIKSDKSAA--GVVNGVRY   322
PGL2 ASPNG  232   -DRSNNVVKNVTIEHSTVSNSENAVRIKTISGAT--GSVSEITY   272
PGU1 YEAST  231   -GRSDNTVNGFWAENNHVINSDNGLRIKTVEGAT--GTVTNVNF   271
      Consensus          v  v v           Ng RIKt  g     G vs i

PGLR LYCES  344   LNVEMQDVKY-PIIIDQNYCDRV-EPCIQQFSAVQVKN   379
PGL1 ERWCA  323   SNVVMKNVAK-PIVIDTVYEKKE-GSNVPDWSDITFKD   358
PGL2 ASPNG  273   SNIVMSGISDYGVVIQQDYEDGK--PTGKPTNGVTIQD   308
PGU1 YEAST  272   ISNKISGIKSYGIVIEGDYLNSK--TTGTATGGVPISN   307
      Consensus     ni m       pi idq Y                s v
```

Fig. 1. A: Domain structure for several PGs. D1, D2, D3, D4 and D5 indicate Domains 1, 2, 3, 4 and 5, respectively. B: Comparison of the deduced polygalacturonase amino acid sequence from *Saccharomyces cerevisiae* (PGU1 YEAST) with those of *Aspergillus niger* (PGL2 ASPNG), *Lycopersicon esculentum* (PGLR LYCES), and *Erwinia carotovora* (PGL1 ERWCA) in Domains 3 and 4. The consensus sequence is indicated below the sequences in each domain. Dark-grey highlights represent that the residues are conserved in all PGs compared, whereas light-grey highlights denote sequences only conserved in some of them.

gardless of their PG phenotype. This result strongly suggested that ORF YJR153w was probably present and conserved in both positive and negative strains. The PCR product size was in close agreement with those observed for other known fungal PGs [3,4].

The PCR product cloned into pBluescript SK+ was amplified for sequencing. The gene was named as *PGU1* and comprises an ORF of 1086 bp. The nucleotide sequence shows complete homology with the ORF YJR153w, thus indicating that this is a

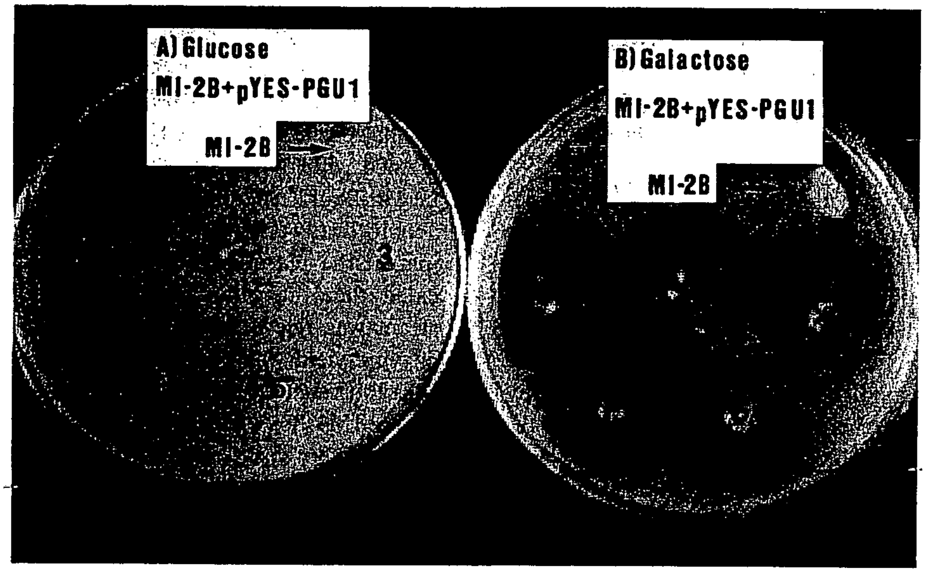


Fig. 2. Production of hydrolysis haloes on plates with polygalacturonic acid by yeast transformants MI-2B+pYES-PGU1 with glucose (A) or galactose (B) as the carbon sources.

highly conserved region in *S. cerevisiae*. The sequence encodes a 361-amino acid polypeptide with a predicted molecular mass of 37 287 Da (Fig. 2) which is in close agreement with the value 36 000 Da previously obtained by gel exclusion chromatography [10]. The first 18 amino acids of the deduced protein represent a potential signal peptide with an alanine residue as the cleaving site. The putative active site is a histidine residue in position 222, which is common for all PGs so far described. The protein has two possible N-glycosylation sites at the residues 318 and 330, which correspond with the typical sequence NxT.

Comparison of the amino acid sequence for yeast polygalacturonase and other PGs from different origins revealed that the protein encoded by *PGU1* gene shares similarity with PGs from bacteria and plants (24%) but more specifically with fungal PGs (54%). The predicted domain structures for these enzymes showed a common C-terminal region where the putative active site (a histidine residue) is located (Fig. 1). This region also includes positively charged residues, which could facilitate protein interaction with the substrate which carries a strong negative charge. An alignment among PGs from different origins including yeast, fungi, plants and bacteria showed the existence of similarity between PGs from yeast and filamentous fungi in all domains. However, with bacteria and plants the identity was only found within the C-terminal region. The similarity between *S. cerevisiae* PG and fungal PGs in Domain 1 was 13%, whereas the similarity for Domain 2 was found to be 64%. The Domains 3 and 4 are common to all sequences compared and contain a highly conserved region (Fig. 1B). The similarity among PGs from all origins was 25% in Domain 3 and 50% in Domain 4. Evolutionary conservation of these domains suggests that they must be essential for the structure and formation of the active site of PGs and may indicate the existence of a common ancestral gene for prokaryotic and eukaryotic PGs.

3.2. Distribution of the cloned gene in S. cerevisiae strains

Southern blot analysis was used to investigate the distribution of the *PGU1* gene in several strains of *S. cerevisiae*. When the *PGU1* gene was used as the probe, a hybridization signal composed of a single band was detected in all strains tested regardless of their PG phenotype (in perfect agreement with PCR results), thus indicating that *PGU1* gene is in only

one copy per haploid genome. No restriction fragment length polymorphism was observed when the genomic DNA was digested with *Hin*dIII.

3.3. Expression of *PGU1* gene in *S. cerevisiae*

In order to confirm that the cloned ORF encodes an active protein, a chimeric plasmid in which the coding region was controlled by either inducible or constitutive promoters was constructed. Thus the *PGU1* gene was cloned into pBEJ16 (containing the constitutive *PGK* promoter) and also into pYES2 as indicated in Section 2. In the latter construct the *PGU1* gene was under the control of the inducible *GAL* promoter. Several strains of *S. cerevisiae* were transformed with these plasmids and tested for the PG phenotype on plates. All transformed strains were able to hydrolize PGA on plates indicating that *PGU1* gene was a structural gene for PG production.

When a PG^- strain (AH22) was transformed with the plasmid pBEJ-PGU1 all the recombinants obtained displayed a positive phenotype, whereas when pYES-PGU1 was introduced in PG^- strains (MI-2B and STX347-1D), the phenotype of the transformants was dependent on the carbon source. They showed a PG^+ phenotype when they were grown in a synthetic medium containing galactose and PG^- when glucose was used as the carbon source (Fig. 2). These results therefore confirm that the *PGU1* gene is the responsible one for the PG phenotype. All the transformants of the PG^+ strain IM1-8b with both recombinant plasmids (pYES-PGU1 and pBEJ-PGU1) exhibited a positive phenotype after 4 days incubation, while the parental strain required at least 6 days.

The enzymatic activity in some strains transformed with both recombinant plasmids was quantified in liquid medium (Table 2). The increase in PG activity was variable (between 2–200 times), depending on the plasmid and strain used. This fact indicated that the genetic background of the receptor strain was relevant for PG production. The highest enzyme yield was obtained with the plasmid pYES-PGU1 (inducible promoter), except in the case of strain IM1-8b which showed higher activity when transformed with plasmid pBEJ-PGU1 than with the inducible one. The low increase in the enzymatic activity in strains MI-2B and IM1-8b could be due to the poor growth exhibited by these strains on galactose when transformed with the pYES-PGU1 construct.

It is important to note that with all the strains and plasmids used the PG activity was enhanced, suggesting that in the negative strains the phenotype may be the result of having a promoter-less *PGU1* gene or else a non-functional one, since we have demonstrated that all strains so far tested contain this structural gene and that they are able to express it under plasmidic promoter control. In addition, the fact that the positive strain 1389-8b showed a very low activity in liquid medium could be due to the effect of shaking, which is an important factor on polygalacturonase activity in yeasts. This aspect

Table 2
Polygalacturonase activity of some *Saccharomyces cerevisiae* strains transformed with the *PGU1* gene

Strain	Carbon source[a]	Enzymatic activity U (μg protein)$^{-1}$
1389-8b	0.5% galactose	0.025
1389-8b+pYES-PGU1	0.5% galactose	5.026
MI-2B	0.5% galactose, 0.5% glucose	0.314
MI-2B+pYES-PGU1	0.5% galactose, 0.5% glucose	1.853
AH22	1% glucose	0.239
AH22+pBEJ-PGU1	1% glucose	1.492
IM1-8b	1% glucose	0.816
IM1-8b+pBEJ-PGU1	1% glucose	9.425
IM1-8b	0.5% galactose, 0.5% glucose	1.382
IM1-8b+pYES-PGU1	0.5% galactose, 0.5 % glucose	2.680

[a]The strains transformed with the plasmid pBEJ-PGU1 were grown with glucose as the carbon source whereas the strains transformed with pYES-PGU1 were grown with galactose; strains MI-2B and IM1-8b were cultivated with galactose and glucose since galactose did not support abundant growth. All strains were incubated with shaking (120 rpm) except strain IM1-8b, that was grown under static conditions.

and the promoter regions of PG will be investigated in the future. Finally, we would like to comment that this enzymatic activity increase (up to 200 times) can be regarded as a significant first stage for over-production PGs for industrial purposes in *S. cerevisiae*. This type of enzymes could possibly be good substitutes for pectinases from fungal origin reported in some cases to contain undesirable enzymes [5,20].

Acknowledgments

This work was supported by the Xunta de Galicia (XUGA20309B94) and by the CICYT (Grant BIO95-1002). The authors thank Dr. Brian Williamson and G. Thow for English correction and specially for critically reading the original manuscript. They also thank Prof. H. Hennecke for his help at the very beginning of the present work. Finally, the authors thank Ana Bonome for her skilful technical assistance throughout. P. Blanco has a grant from the University of Santiago de Compostela.

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